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RFLP-based phylogenetic analysis of wide compatibility varieties in *Oryza sativa* L.

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Abstract Twenty-one wide compatibility varieties (WCVs) of rice together with three *indica* and three *japonica* testers were assayed with 160 DNA probes that were selected to represent the entire RFLP map at an average interval of 11 cM. On the basis of four enzyme digestion 125 probes detected polymorphisms among the WCVs and subspecies' testers. Among these polymorphic probes there were 68 that could distinguish the *indica* from the *japonica* testers. Two dendrograms were constructed on the basis of 398 polymorphic fragments of 125 probes and 139 polymorphic fragments of 68 subspecies' differentiating probes in combination with single enzymes, respectively. The reliability and representativeness of the testers and the levels of DNA variations among WCVs were estimated. The potential of WCVs in the utilization of intersubspecific heterosis is discussed.

Key words *Oryza sativa* L. · RFLP · Wide compatibility · Subspecies differentiation · Heterosis

Introduction

Hybrid rice has been a great success in China. To further improve rice production, the utilization of *indica-japonica* hybrids has been proposed as a very high degree of heterosis in many intersubspecific crosses has been observed (Zeng et al. 1980; Yuan 1987). The main obstacle in utilizing intersubspecific heterosis in rice has been the partial reproductive barrier between the subspecies.

The discovery of wide compatibility varieties (WCVs) which produce fertile F_1 plants when crossed to *indica* as well as *japonica* varieties has opened the door to solving this semi-sterility problem. The genetic mechanism of wide compatibility has been studied, and the WC locus S_5 has been located on chromosome 6 between the *C* (chromogen for pigmentation) and *wx* (waxy endosperm) loci (Ikehashi and Araki 1986).

Recently, rice breeders in China have screened a number of WCVs from different origins, which provide the sources of wide compatibility. These can be used either directly as the parents of intersubspecific hybrids or as donors of this trait for cultivars that are to be used as the parents of the intersubspecific hybrids. In transferring this gene, however, selection in a segregating population can be carried out only after comparision of the spikelet fertilities of F_1 hybrids of individual progenies to those of the standard subspecies' testers. This is time-consuming and tedious work. Furthermore, it is expected that hybrids resulting from crosses between WCVs with an *indica* background and *japonica* cultivars, or WCVs with a *japonica* background and *indica* cultivars, will show stronger heterosis than when WCVs with an *indica* background are crossed with *indica* cultivars, and vice versa. Clarification of phylogenetic relationships among these WCVs is therefore important for the predictability of intersubspecific heterosis.

The development of DNA restriction length polymorphism (RFLP) technology provides a new tool by which to study the genetic mechanism of wide compatibility and genetic variations in WCVs. Recently we tagged a wide compatibility gene via linkage to RFLP markers (Zheng et al. 1992) in a three-variety cross, WCV/*indica*//*japonica*. In the same segregating population, a heading date gene was also tagged with RFLP markers (Shen et al. 1994). These tagged genes may be used effectively in genetic studies and breeding practices.

In the study reported here RFLP analysis has been used to estimate the reliability and representativeness of the plant breeder-adopted subspecies' testers and the level of DNA variations among 21 WCVs. All of the testers and WCVs were assayed with a total of 160 probes that had been selected to represent the whole rice RFLP map (Rice molecular map, September, 1989, from S. D. Tanksley's lab) at an average interval of 11 cM. Two dendrograms were constructed on the basis of RFLP variations revealed by either 125 polymorphic probes or by 68 subspecies-differentiating probes (in combination with a single restriction enzyme), respectively.

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Table 1 List of *Oryza sativa* varieties included in study

	Varieties	Type[a]	Origin
1	T984	jap	Zhejiang, China
2	T986	jap	Zhejiang, China
3	Pecos	jap	USA
4	Nekken 1	jap	Japan
5	02428	jap	Jiangsu, China
6	Zhe-hu 114	jap	Zhejiang, China
7	Zhe-hu 157	jap	Zhejiang, China
8	3264	jap	Zhejiang, China
9	CPSLO 17	jav	USA
10	CPSLO 19	jav	USA
11	Lun-hui 422	jav	Hunan, China
12	Pei-ai 64	ind	Hunan, China
13	Ketan Nangka	jav	Indonesia
14	Akenohoshi	jap	Japan
15	L201	ind	USA
16	Lemont	jav	USA
17	Suweon 283	ind	Korea
18	Bellemont	jav	USA
19	Dourado precoce	jap	Italy
20	IR58	ind	Philippines
21	Dular	Aus	India
22	Ballila	jap	Italy
23	Akihikari	jap	Japan
24	Zao-sha-keng	jap	Jiangsu, China
25	Nan-te-hao	ind	Jiangxi, China
26	Nanjing 11	ind	Jiangsu, China
27	IR36	ind	Philippines

[a] jap, *Japonica*; ind, *indica*; jav, *javanica*

Materials and methods

Plant materials

Rice seeds of the 27 varieties were provided by the Germplasm Department and Genetics and Breeding Department of the China National Rice Research Institute, Hangzhou, China and are listed in Table 1. The classifications used in the table refer to integrated classic concepts and are generally accepted by plant breeders. Varieties '22–24' and '25–27' are typical *japonica* and *indica* varieties, respectively, which were proposed as standard testers for screening WCVs in China in 1987 (Gu et al. 1991). Leaves were harvested for DNA extraction from a single plant of each variety grown in the paddy field during the normal growing season in Hangzhou.

RFLP probing

Total DNA extraction, restriction endonuclease digestion, electrophoresis, Southern blotting, hybridization and autoradiography have been described previously (Zheng et al. 1990; Lu and Zheng 1992). The restriction endonucleases *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I were used to digest the total DNA. Altogether, 160 probes were used, including 149 rice random genomic clones (RG), 2 rice cDNA clones (RZ) and 7 oat cDNA clones (CDO), all of which were a gift from Dr. S. D. Tanksley of Cornell University, and 2 rice genomic clones (G) from Dr. H. Uchimiya of the Institute of Applied Microbiology, University of Tokyo (Table 2). All of the RG clones were selected from the RFLP map (September, 1989) of Dr. S. D. Tanksley's lab except for 4 clones that had not yet been mapped. RZ and CDO clones were from the 1991 version of the map (McCouch and Tanksley 1991). The two G clones are *indica*-specific clones; one, G318, has been mapped on chromosome 12 and the other, G93, has not yet been mapped (Oba et al. 1991; H. Uchimiya personal communication). The average interval between probes was about 11 cM (mainly based on the Dr. S. D. Tanksley's RFLP map of 1989). The majority of these clones represent single-copy sequences.

Data analysis

Each hybridizing fragment detected by Southern analysis was treated as a unit character. All of the polymorphic hybridizing fragments detected among the 27 varieties were used to make comparisons between varieties. Genetic distances between varieties were quantified according to Nei (1987, formula 5.53–5.55) using a BASIC program developed in our laboratory. Because RFLPs detected by the same probe but different restriction enzymes may not represent independent mutation in the rice genome, only polymorphic fragments based on single restriction enzymes were used for the calculation of genetic distances. Among the total 125 polymorphic probes, 68 probes produced hybridization patterns that were identical for the three *indica* and three *japonica* testers, respectively. We considered them as *indica-japonica* differentiating probes. In addition to the two G clones mentioned above, RG358 also only hybridized to the DNA of *indica*. We consider all three to be *indica*-specific probes. For comparison, two dendrograms were constructed using the unweighted paired-group method with arithmetic mean (UPGMA) (Sokal and Michener 1958) based on all 125 polymorphic probes and the 68 subspecies-differentiating probes, respectively.

Results and Discussion

As shown in Table 2, 125 out of 160 probes detected polymorphism among the 27 varieties on the basis of four enzyme digestions. In most cases, a given probe that detected polymorphisms with one enzyme also detected polymorphism with other enzymes. This situation arises because RFLPs in rice are believed to be caused by insertion/deletions (McCouch et al. 1988), which implies that RFLPs detected by the same probe but different restriction enzymes may not represent independent mutations. Therefore, we did not use all of the polymorphic probe-enzyme combinations in our analysis, but only those polymorphic probes based on single enzymes. Figure 1a shows a probe detecting polymorphism among the varieties. There were no obvious differences in the distribution of polymorphic probes on different chromosomes, even though the percentage of polymorphic probes on any one chromosome ranged from 54% to 100% (Table 2).

Significant differentiating between *japonica* and *indica* cultivars was detected. Among the 125 polymorphic probes, there were 68 probes that produced identical hybridization patterns for testers within subspecies but different patterns between subspecies (Fig. 1b). We define these as subspecies-differentiating probes. RG358, like the G clones, hybridized readily to the DNAs of *indica* varieties, but hardly at all to the DNAs of *japonica* varieties. A possible explanation of why these clones hybridized only with *indica* DNAs is that the RG clones were derived from an *indica* ('IR36') library and the 2 G clones were also derived from an *indica* cultivar (culture 340). A similar phenomenon was observed by McCouch et al. (1988) when screening *indica* and *japonica* germ plasm with the same probes. We consider clones with null alleles in the *japonica* genome to be *indica*-specific clones, and they could be regarded as being extreme examples of subspecies-differentiating probes. The fact that no *japonica*-specific clones were found using *indica*-derived clones in our experiment is reasonable. All of the differentiating probes were randomly distributed on different chromosomes (Table 2).

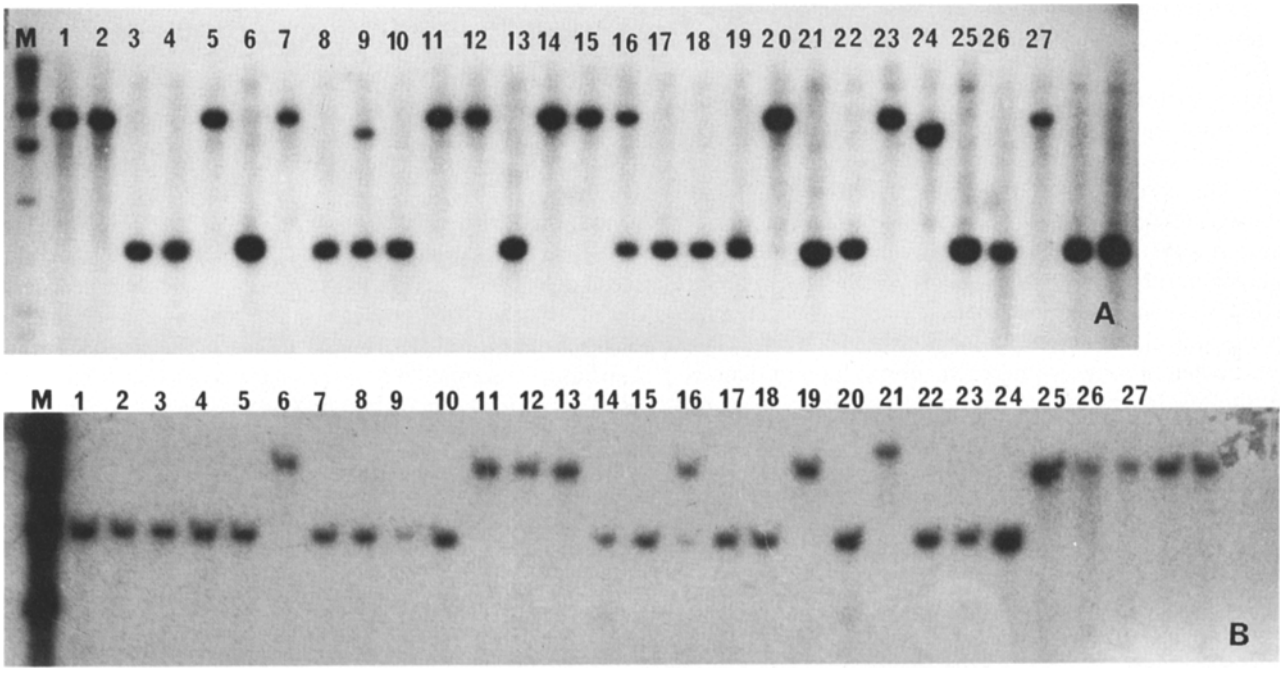
Dendrogram A was constructed according to genetic distances based on 398 polymorphic fragments of 125 probes

Table 2 Probes used and their performances in this experiment

Linkage group	Probes[a]	Number of probes	Number of polymorphic probes (%)	Number of differentiating probes
1	RG101, RG132, RG220, RG222, RG246, RG345, RG350, RG374, RG381, RG394, RG400, RG462, G472, RG532, RG536, RG541, RG690, RG755, RG780, CDO251, CDO281, CDO345	22	19 (86)	10
2	RG25, RG83, RG95, RG102, RG144, RG152, RG157, RG171, RG252, RG256, RG322, RG324, RG437, RG520, RG654, RG752, CDO32, CDO196, DO204	19	14 (77)	8
3	RG96, RG100, RG104, RG224, RG227, RG348, RG393, RG409, RG450, RG482, RG722, RG745 ,G944	13	7 (54)	4
4	RG122, RG163, RG169, RG214, RG329, RG454, G620, RG864, CDO456	9	6 (67)	3
5	RG13, RG119, RG182, RG207, RG313, RG346, RG403, RG435, RG470, RG474, RG493, RG556, RG573, RZ70	14	11 (79)	5
6	RG64, RG123, RG138, RG147, RG162, RG172, G213, RG244, RG408, RG424, RG456, RG648, RG653, RG716, RG778, RZ405	16	11 (67)	8
7	RG29, RG30, RG156, RG173, RG351, RG417, RG511, RG650, RG678, RG711, RG769, RG811	12	11 (92)	4
8	RG20, RG28, RG108, RG333, RG365, RG555, RG598, RG634, RG885, RZ66	10	10 (100)	4
9	RG125, RG136, RG141, RG358, RG432, RG451, RG553, RG570, RG662, RG667, RG757, r45s	12	9 (75)	6
10	RG134, RG241, RG257, RG561	4	3 (75)	2
11	RG2, RG16, RG103, RG118, RG167, RG303	6	5 (83)	3
12	RG9, RG81, RG91, RG98, RG181, RG235, RG304, RG323, RG341, RG396, RG449, RG457, RG543, RG788, RG869, RG901, RG958, G318	18	16 (89)	9
Unmapped	RG382, RG385, RG608, RG684, G93	5	3 (60)	2
Total		160	125	68

[a] Probes that are underlined refer to subspecies-differentiating probes

Fig. 1A, B Autoradiograms derived from hybridizing single-copy clones onto DNA from 21 WCVs, 3 *japonica* and 3 *indica* testers (1–27, see Table 1 for details). *M* Lamda-*Hin*dIII molecular weight. **A** *Hin*dIII digest probed with RG711; **B** *Eco*RI digest probed with RG256. The fragments of three *japonica* testers (*lanes 22–24*) showed the same size, while those of three *indica* testers (*lanes 25–27*) also were of the same size, but different from those of the *japonica* testers



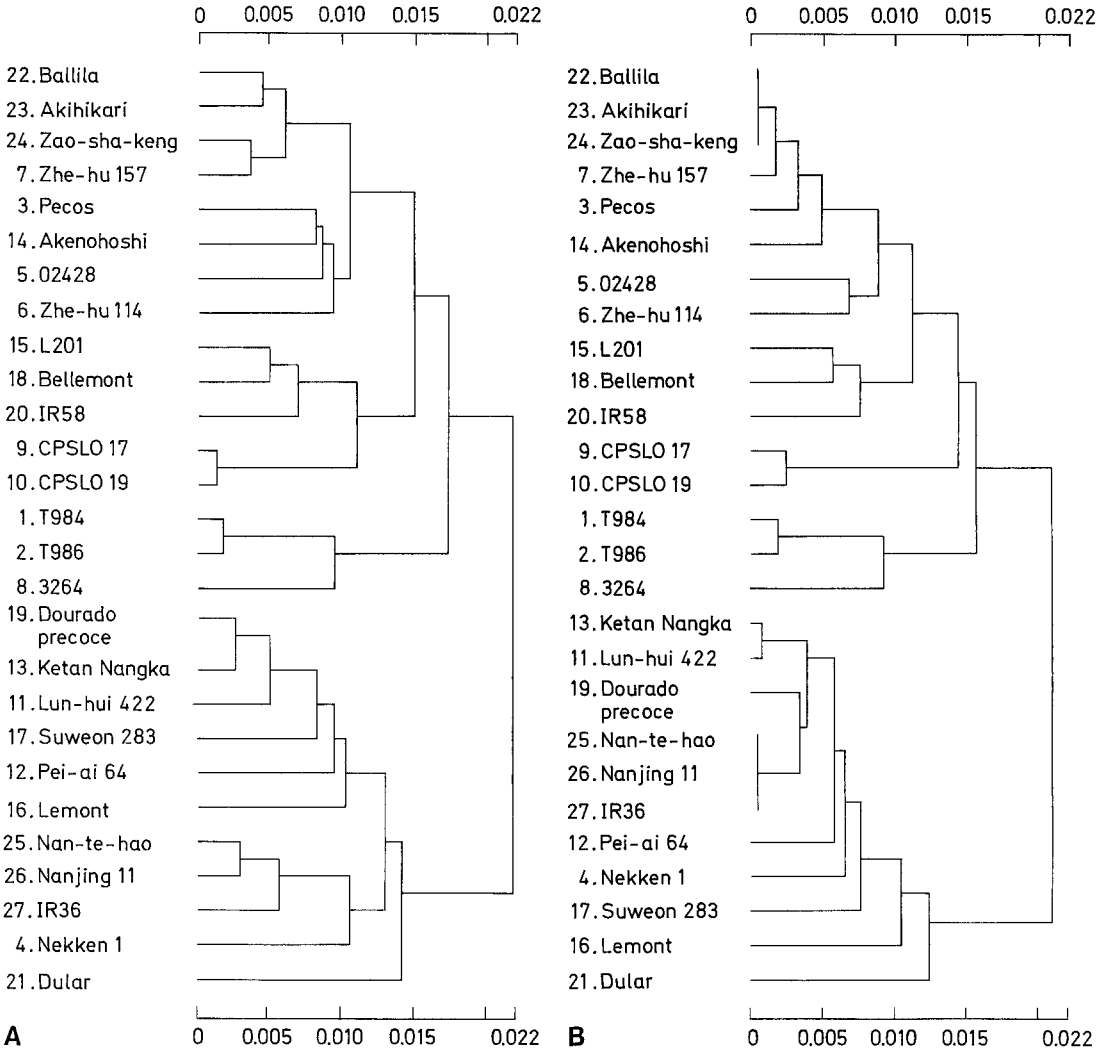


Fig. 2A, B Dendrograms of 21 WCVs, three *japonica* testers and three *indica* testers of *Oryza sativa* by RFLP analysis. The *numbers below* and *above* the dendrograms are genetic distance scales according to Nei (1987). **A** A dendrogram constructed on the basis of 398 polymorphic fragments of 125 probes in combination with single enzymes. **B** A dendrogram constructed on the basis of 139 polymorphic fragments of 68 subspecies' differentiating probes in combination with single enzymes

with a single enzyme, while dendrogram B was based on 139 polymorphic fragments of 68 subspecies-differentiating probes with a single enzyme (Fig. 2). These two dendrograms show similar phylogenetic relationships among subspecies' testers and WCVs.

The proportion of shared fragments and genetic distances between genotypes of *indica* and *japonica* testers based on 125 polymorphic probes with a single enzyme are shown in Table 3. The genetic distances between testers of different subspecies are much larger than within each subspecies, guaranteeing their reliability for representing the differentiation between subspecies. On the other hand, the proportion of shared fragments observed between testers within each subspecies was about only 40% for both sets of testers. This indicates differentiation within each subspecies and demonstrates the reliability of the testers included in this study for representing that variation. Testers of the respective two subspecies cluster together, in dendrogram A, further indicating that they are reliable as subspecies' testers in screening for WCVs. In compatibility studies of rice, Ikehashi and Araki (1984) used 'Akihikari' and 'Nihonmasari' as *japonica* testers and 'IR36' as an *indica* tester, while at the International Rice Research Institute (IRRI) 'Akihikari', 'Toyonishiki' and 'Taichung 65' were used as *japonica* testers, 'IR36', 'IR50' and 'IR64' as *indica* testers (cited by Gu et al. 1991). In China, a different set of subspecies' testers has been established, and compatibility has been studied on the F_1 fertility between that

Table 3 RFLP-based genetic distances between testers of different subspecies and within subspecies

	Between subspecies	Within *indica*	Within *japonica*
Proportion of fragments shared	0.181 ± 0.022	0.420 ± 0.024	0.412 ± 0.017
Genetic distance	0.0611 ± 0.0079	0.0098 ± 0.0033	0.0110 ± 0.0023

set of *indica* and *japonica* testers. F_1 fertility differences were found when different *indica* testers were crossed to *japonica* testers and vice versa. This also indicates the differentiation of testers within each subspecies (Gu et al. 1991).

From the point of view of plant breeders, a WCV is defined when (1) the average F_1 fertility of crosses between a WCV and *indica* or *japonica* tester is significantly higher than the F_1 fertility of crosses between *indica* and *japonica* testers or (2) the fertility of most F_1 hybrids derived from crosses between a WCV and a wide range of typical *indica* and *japonica* varieties are normal (>65%) (Min et al. 1990). Nevertheless, such approaches based on F_1 fertility do not provide enough information to reveal the genetic differences between WCVs and typical *indica* and *japonica* testers. Indeed, the genetic distances between some WCVs, such as T984 and T986, and *indica* and with *japonica* testers are similar. It will not be surprising that such varieties demonstrate a reasonable compatibility with both *indica* and *japonica* testers, even if they do not possess any gene for wide compatibility. Moreover, as mentioned above, heterosis depends on the genetic differences between parents so that only WCVs with large genetic distances from either *indica* or *japonica* testers will be of great potential importance in breeding practices.

In dendrogram B, the three *japonica* testers are identical, as are the *indica* testers. The grouping of all WCVs is in keeping with that in dendrogram A. The number of probes for dendrogram B is only half that in dendrogram A. Subspecies-specific probes can be used more effectively for phylogenetic analysis in this respect.

In the study reported here, we used RFLP data based on 125 polymorphic probes with a single enzyme to ensure complete coverage of the whole rice genome and to avoid duplication and overlap if the RFLPs were from insertion/deletion. However, if the RFLP is caused by point mutations, each probe-enzyme combination would represent an independent mutation, and all of the data derived from probing DNA digested with several enzymes could be used.

Rice breeders have used morphological traits, F_1 fertility in remote crosses, isozyme electrophoresis and numerical taxonomy for classifying subspecies (Zhou et al. 1988). RFLP analysis provides an effective tool by which to minimize the ambiguities inherent in subspecies' classification obtained by previous methods. Since the differentiation of *indica* and *japonica* is significant at the DNA level, it is possible to use a set of subspecies-differentiating probes for classification within *Oryza sativa*. We are now trying to use fewer probes and are testing more *indica* and *japonica* varieties in an effort to establish such a set.

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